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**(54) Title:** INTRACELLULAR DELIVERY OF BIOLOGICALLY ACTIVE SUBSTANCES BY MEANS OF SELF-ASSEMBLING LIPID COMPLEXES**(57) Abstract**

Disclosed are methods and compositions for facilitating intracellular delivery of biologically active substances of pharmaceutical agents, comprising self-assembling complexes of positively and negatively charged lipid species capable of interacting with a substance to be delivered. The assembled complexes have a net positive charge suitable for spontaneously attaching to negatively charged cell membranes, and may comprise a neutral or positively charged bioactive substance first encapsulated in or complexed with negatively charged lipid vesicles which are next complexed with cationic lipid vesicles having a net positive charge before or in the process of administration to the cell.

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INTRACELLULAR DELIVERY OF BIOLOGICALLY ACTIVE SUBSTANCES  
BY MEANS OF SELF-ASSEMBLING LIPID COMPLEXES

Background of the Invention

5       The present invention relates to methods that are used  
to enhance delivery of biologically and pharmacologically  
active agents, particularly polynucleotides, proteins,  
peptides, and drug molecules, by facilitating transmembrane  
transport or by encouraging adhesion to biological surfaces.  
10   It relates particularly to self-assembling systems,  
comprising lipids having cationic charged regions, that  
facilitate intracellular delivery of these bioactive agents.

Not all bioactive agents need to enter cells to exert  
their biological effect. Some are able to act on cell  
15   surfaces through cell surface receptors, while the effect of  
others is mediated through an interaction with extracellular  
components. However, many valuable therapeutic agents that  
are most effective in influencing cell function at the  
subcellular or molecular level, comprising natural  
20   biological molecules and their analogues, or foreign  
substances such as drugs, are preferably incorporated  
within the cell in order to produce their effect. In many  
cases, the cell membrane provides a selective barrier which  
is impermeable to many of these agents.

25       The complex composition of the cell membrane comprises  
phospholipids, glycolipids, and cholesterol, as well as  
intrinsic and extrinsic proteins, and its functions are  
further influenced by cytoplasmic components, comprising  
small ions and subcellular structures. Interactions among  
30   these elements and their response to external signals make  
up transport processes responsible for membrane selectivity  
of various cells. Successful intracellular delivery of  
agents not naturally taken up by cells has been achieved by  
exploiting the natural process of membrane fusion or by  
35   exploiting the cell's natural transport mechanisms which  
include endocytosis and pinocytosis (Duzgunes, N.,  
Subcellular Biochemistry 11: 195-286 (1985)).

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The intracellular delivery of bioactive agents is essential for many useful applications. The delivery of polypeptides, for example, could be useful in therapies to correct genetic defects, for immunization, or in the treatment of various other disorders. Intracellular delivery of beneficial or interesting proteins can be achieved by introducing expressible DNA and mRNA into the cells of a mammal, a useful technique termed transfection. Gene sequences introduced in this way can produce the corresponding protein coded for by the gene by using endogenous protein synthetic enzymes. Therapeutic peptides that could be introduced in this way include lymphokines, such as interleukin-2, tumor necrosis factor, the interferons, growth factors, such as nerve growth factor, epidermal growth factor, and human growth hormone, tissue plasminogen activator, factor VIII:C, erythropoietin, insulin, calcitonin, and amylin. In addition, major therapeutic benefits could be obtained by means of intracellular delivery of toxic peptides such as ricin, diphtheria toxin, or cobra venom factor in order to eliminate diseased or malignant cells. Transfection of cells has been carried out by various methods, comprising calcium phosphate precipitation, or DEAE dextran or electroporation methods. Each of these methods is restrictive in some way and none is highly efficient.

Efforts to develop more satisfactory methodologies for delivering functional polynucleotides into living cells have continued steadily over the years. A major advance was the development of cationic lipid technology, based on a discovery that a positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), in the form of liposomes, or small vesicles, could interact spontaneously with DNA, which is negatively charged, or anionic, to form lipid-DNA complexes having a net positive charge and capable of fusing with the negatively charged cell membranes of tissue culture cells, to achieve both uptake

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and expression of the DNA (Felgner, P.L. et al. Proc. Natl. Acad. Sci., USA 84:7413-7417 (1987) and U.S. Patent No. 4,897,355 to Eppstein, D. et al.). The Lipofectin™ reagent (Bethesda Research Laboratories, Gaithersburg, Maryland),  
5 an effective commercial cationic lipid reagent for the delivery of highly anionic polynucleotides into living tissue culture cells, comprises positively charged DOTMA liposomes.

Since the introduction of DOTMA, other cationic lipid  
10 agents have been developed for use in transfecting cells. A DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonio)-propane (DOTAP) has been successfully used in combination with a phospholipid to form DNA-complexing vesicles (Stamatatos, L. et al., Biochemistry 27(11):3917-  
15 3925(1988). Also a co-pending U.S. Patent Application Cationic Lipids for Intracellular Delivery of Biologically Active Molecules, filed April 19th, 1990 by Felgner et al., discloses cationic lipids which are more effective and less toxic than presently known cationic lipids for  
20 transfection. Cationic lipid methodology is presently preferred over other methods; it is more convenient and efficient than calcium phosphate, DEAE dextran or electroporation methods. However, it can only be applied to polyanionic molecules, such as DNA. Unfortunately, not  
25 all bioactive substances of interest are as negatively charged as polynucleotides; therefore, the extension of cationic lipid methodology to the delivery of other molecules, for example, many proteins, is not feasible.

Transport of proteins is among the most selective and  
30 specific of transmembrane processes. Such processes involve protein-protein recognition (signal sequences), frequently require ATP, or are enzymatically driven. Viruses have proteins capable of accessing these processes successfully, thus allowing viral infections of cells to  
35 occur. The viral method of intracellular penetration has been exploited to develop a methodology which uses viral "chimerasomes" or proteoliposomes for intracellular protein

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delivery. Viral chimerasomes are lipid vesicles containing molecules intended for intracellular delivery together with two critical proteins isolated from viruses. One of these proteins is a cell surface recognition protein which promotes attachment of lipid vesicles with the cell surface. Another is a viral fusion protein which facilitates the introduction of the viral contents into the cell cytoplasm. Unlike the Lipofectin<sup>™</sup> reagent, the chimerasome methodology is broadly applicable to the delivery of proteins as well as polynucleotides. However, it is more expensive and difficult to manufacture than cationic lipid reagents because it involves isolation and purification of components of potentially infectious virus particles. Stability of the proteins and lipids in the chimerosome reagent is a potential problem, and it is also somewhat less convenient to use than the cationic lipids.

A modification of the cationic lipid technology has been suggested which may confer a degree of specificity on its transfection procedures similar to that of chimerosomes. According to the strategy of Eppstein, D. et al. U.S. Patent No. 4,897,355, polynucleotide molecules are first treated with DOTMA to produce a positively charged complex. This complex is then treated with negatively charged liposomes to form a double coated, negatively charged complex. The negatively charged coat may comprise a coupling reagent. Receptor specific agents, such as antibodies, lectins, and other ligands may then be attached to the negatively charged outer layer through the coupling reagent. The overall negative charge of the double-coated complex prohibits its non-specific interaction with the negatively charged membranes of cells while allowing relatively specific interaction through the binding of integral ligands. The defect of this approach, like that of the chimerosome methodology, is its relative complexity, and its requirement for specific binding reagents; further, its application, like that of the parent cationic lipid methodology, is limited to polyanionic materials, that is,

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polynucleotides and negatively charged proteins.

It would be desirable to have an intracellular delivery system having the advantages of the above described cationic lipid and chimerosome methodologies but  
5 without their inherent limitations.

It is therefore an object of the invention to provide a methodology for the intracellular delivery of a wide variety of bioactive agents, particularly proteins, regardless of net charge.

10 The intracellular delivery systems disclosed herein exploit self-assembling processes found in natural systems to join together molecular subcomplexes through physical mechanisms of ionic attraction and repulsion and hydrophobic-hydrophilic interactions. The most pertinent  
15 example of such systems in nature is the simple assemblies of lipids and proteins to form cell membranes. The physical forces holding such structures together are relatively weak and non-specific, and this, among other factors, allows the assembly to proceed spontaneously and  
20 rapidly.

An advantage of self-assembling systems that imitate these natural systems is that the energy conserving nature of their assembly and disassembly eliminates the necessity of chemically bonding the subunits together or providing  
25 means to degrade them to the active units in vivo. The property of self-assembly therefore enables a convenient and practical manufacturing procedure.

It is also an object of the invention to provide a generally applicable intracellular delivery system which is  
30 self-assembling, eliminating the need for synthesis procedures.

It is further an object of the invention to provide reliable and efficient reagents for an intracellular delivery system which are commercially feasible, that is,  
35 relatively easy and inexpensive to manufacture and having an extended shelf life.

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Brief Description of the Drawings

The following figures show the intracellular delivery of proteins into tissue cells in culture according to the methods of the invention. Each figure consists of a pair of photomicrographs produced by (a) phase contrast and (b) fluorescence microscopy.

Figure 1 shows intracytoplasmic delivery of fluoresceinated dextran (MW 4000), into NIH-3T3 cells.

Figure 2 shows intracytoplasmic delivery of fluoresceinated dextran (MW 4000) into HELA cells.

Figure 3 shows the intracellular delivery of Rhodamine-PE into NIH-3T3 cells.

Figure 4 shows the delivery of a low molecular weight dye, 6CF, into NIH-3T3 cells.

Figure 5 shows the delivery of Rhodamine-phalloidin into NIH-3T3 cells.

Summary of the Invention

The invention provides a method for making a vehicle for administering a bioactive substance to a cell, comprising the steps of providing the substance in a first lipid vesicle which vesicle comprise at least one negatively charged lipid and is negatively charged; and combining this first lipid vesicle with second lipid vesicles comprising at least one cationic lipid and having a net positive charge, whereby the first negatively charged vesicles are coated with at least one positively charged lipid vesicle. The bioactive substance can be encapsulated in the vesicle by containment in the aqueous core; alternatively, if the bioactive substance is lipophilic or has a lipophilic or amphipathic region, it may be incorporated by means of that lipophilic structure into the lipid wall of the vesicle.

In another embodiment, there is provided a method for making a vehicle for administering a bioactive substance to a cell, comprising the steps of forming a first complex



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comprising a positively charged macromolecule and at least one lipid vesicle, wherein the lipid vesicle comprise a negatively charged lipid and has a net negative charge; and contacting the first complex with lipid vesicles comprising at least one cationic lipid and having a net positive charge to form a second complex.

According to another aspect of the invention, there is provided a method for administering a bioactive substance to a cell, comprising the steps of providing the substance in a negatively charged lipid vesicle; and administering the negatively charged lipid vesicle to a cell together with a lipid vesicle comprising at least one cationic lipid and having a net positive charge.

In another embodiment there is provided a method for administering a bioactive substance to a cell, comprising the steps of preparing a complex comprising said bioactive substance and a negatively charged lipid vesicle; and administering the complex to a cell together with a lipid vesicle comprising at least one cationic lipid and having a net positive charge. The methods of administering bioactive substances to a cell can be applied in vivo as well as in vitro.

The invention also provides a composition of matter, comprising a bioactive substance contained in a negatively charged lipid vesicle and lipid vesicles, comprising at least one cationic lipid species and having a net positive charge, at least partially covering the negatively charged lipid vesicle. In preferred embodiments, the composition has a net positive charge. The bioactive substance of the composition can be a biological macromolecule, such as, for example, a polynucleotide, a protein or polypeptide. The bioactive substance may also be a drug, and the drug may be a small organic molecule. According to one preferred embodiment, a bioactive substance is encapsulated within a negatively charged lipid vesicle. The bioactive substance may also be lipophilic or comprise a lipophilic region, and is thereby incorporated among the lipids of the

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lipid vesicle.

The invention also provides a kit, comprising materials, supplies, reagents, and instructions for preparing self-assembling lipid complexes for the delivery of a bioactive substance to a cell, either *in vitro* or *in vivo*, comprising at least one vessel containing at least one negatively charged amphipathic lipid species; and a quantity of a lipid substance, the substance comprising at least one positively charged amphipathic lipid species. The kit can further comprise at least one hydrating buffer solution and an instruction leaflet.

In any of the methods or compositions disclosed suitable positively charged lipid species can be known or unknown cationic lipids, such as, for example DOTAP or DOTMA or can have the structure of any of the novel cationic lipids described herein.

#### Detailed Description of the Invention

The present invention provides vehicles, comprising self-assembling lipid complexes, capable of delivering bioactive substances, particularly polynucleotides, oligonucleotides, proteins, peptides or drugs into living cells in a convenient and efficient manner. According to the methods presented, lipid vesicles, comprising lipid species having either a negative or positive charge, are combined with the substance to be delivered in a properly ordered sequence, forming self-assembling lipid complexes containing the substance to be delivered and having a positively charged outer lipid layer.

The invention, like cationic lipid technology, takes advantage of the natural fusion properties of a positively charged lipid to promote passage of bioactive substances associated with that lipid across the negatively charged cell membrane. However, the invention provides a prior association of the bioactive substance with negatively charged lipids to form primary complexes having a net

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negative charge. This primary complex promotes a subsequent secondary complex formation with the liposomes comprising the cationic lipids that facilitate membrane transport. In this way the invention extends cationic lipid methodology to all bioactive agents intended for intracellular delivery, regardless of size or net charge. It also provides an intracellular delivery process that is quite non-specific and is therefore broadly applicable to all cell types, in cases where these substances, as such, are not able to penetrate the cell membrane, including those of cells that do not contain specific ligand or antibody receptors.

#### Lipid Vehicles for Intracellular Delivery

Vehicles for intracellular delivery according to the invention comprise a primary negatively charged lipid complex in association with the bioactive substance to be delivered and a secondary complex, having a net positive charge and comprising the primary negative complex in association with positively charged liposomes. The invention provides two general strategies for assembling the primary negatively charged lipid complex comprising the bioactive substance. According to a preferred strategy, the bioactive substance to be delivered is entrapped within a lipid vesicle or liposome comprising negatively charged lipids and having a net negative charge. The entrapment can occur, according to one embodiment of the invention, as an incorporation of a lipophilic bioactive substance, or such a substance having a lipophilic region, into the wall of the lipid vesicle. According to another embodiment, the entrapment can occur as an encapsulation of hydrophilic bioactive substance into the aqueous core of the lipid vesicle. For both lipophilic and hydrophilic substances, the uptake into the negatively charged liposome occurs during the process of liposome formation, typically the hydration of a lipid film, disclosed herein, and illustrated by examples. Examples 3, 5, and 6 disclose the encapsulation of substances within negatively charged

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liposomes, and Example 4 discloses the incorporation of a lipid derivative into the liposome wall.

According to another strategy, the bioactive substance is contacted with negatively charged liposomes, and these liposomes then at least partially enclose the substance to provide a primary lipid complex having negatively charged regions. In a preferred embodiment, the bioactive substance has a net positive charge and interacts with the negative liposomes by means of charge attraction; alternatively, the bioactive substance can be neutral or negatively charged but comprise lipophilic regions that promote its association with the liposomes of the complex through hydrophobic or lipophilic interactions. In yet another embodiment, the bioactive substance can comprise amphipathic peptide helices, for example of the type disclosed by Segrist, U.S. Patent No. 4,643,988, that can attach to the lipid vesicle surface through hydrophobic and lipophilic interactions, thus anchoring the molecules of which they are a region.

In other preferred embodiments, the negatively charged liposomes used to form the complex may contain the same or other bioactive substances, either encapsulated or incorporated, as disclosed above.

The second element of the intracellular delivery system of the invention comprises liposomes formed from positively charged cationic lipids and having a net positive charge. On contact with the primary complex, comprising either the negatively charged liposomes or the negatively charged lipid complex, the positively charged liposomes spontaneously form complexes having a net positive surface charge.

In all the embodiments described above, the positively charged assembled complexes, when placed in contact with cells, will spontaneously attach to cell surfaces and interact directly with the plasma membrane, according to conventional cationic lipid methodology. Delivery of the bioactive cargo into the cell cytoplasm can occur by various

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mechanisms. For example, the lipids of the complex may either directly fuse with the plasma membrane and discharge the entrapped substance intracellularly; alternatively, it may be phagocytized and interact with other internal  
5 membranes or the membranes of the phagocytic compartment itself.

All of these assemblies, comprising the encapsulation or incorporation of substances within lipid vesicles, the association of substances with positively or negatively  
10 charged lipids or vesicles thereof, the association of positively and negatively charged lipids and vesicles thereof, and even the attachment of the vesicles to the target cell surface and fusion directly with cell membranes, occur spontaneously as the result of repulsions  
15 and attractions of ionic charges and hydrophobic-hydrophilic interactions.

Therefore, the negative and positively charged lipid complexes form almost instantaneously, and accordingly the primary negatively charged complexes can be contacted with  
20 the positively charged liposomal preparation and target cells simultaneously. Alternatively, the primary negative complexes and positively charged liposomes may be mixed to form secondary complexes before contact with the target cells. For *in vivo* applications, prior complex formation  
25 is preferred, while for *in vitro* applications, the positively charged empty liposomes are conveniently added to a cell culture at the same time as the negatively charged liposome-bioactive agent complexes, thus avoiding a separate mixing step.

### 30 Liposome Formulations

The lipid reagents of the invention may comprise lipid mixtures similar to that of the physiological cell membrane, comprising phospholipids as primary components. The lipid reagents can further comprise any of the  
35 conventional synthetic or natural liposome materials, including phospholipids from natural plant or animal sources such as phosphatidylcholine,

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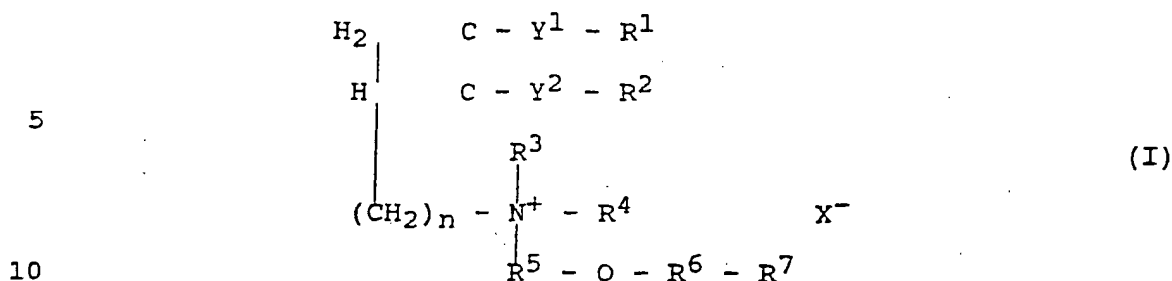
phosphatidylethanolamine, sphingomyelin, phosphatidylserine, or phosphoinositol. Synthetic phospholipids that may also be used include, but are not limited to, dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine and the corresponding synthetic phosphatidylethanolamines and phosphatidylglycerols. Other additives such as cholesterol, glycolipids, fatty acids, sphingolipids, or gangliosides can also be used, as is conventionally known for the preparation of liposomes.

The positively and negatively charged lipid vesicles used in the methods of the invention are typically prepared as appropriate from a mixture of either cationic lipids or negatively charged lipids, neutral lipids and cholesterol or a similar sterol. Neutral lipids can be phosphatidylcholine, phosphatidyl ethanolamine, similar phospholipid analogs, or mixtures of these, as well as monoglycerides, diglycerides and triglycerides.

The negatively charged lipid reagents of the invention are those comprising at least one lipid species having a net negative charge at physiological pH or combinations of these. Suitable lipid species comprise phosphatidyl glycerol and phosphatidic acid or a similar phospholipid analog. The positively charged lipid reagents of the invention are those comprising at least one cationic lipid species having a net positive charge at physiological pH. Suitable lipid species comprise known cationic lipids, such as 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) or N-( $\omega$ ,  $\omega$ -1-dialkoxy)-alk-1-yl-N, N, N-trisubstituted ammonium surfactants, such as DOTMA, or complex cationic lipids having similar structures and properties or mixtures of these. Particularly preferred cationic lipids are those disclosed in a co-pending U.S. Application entitled Cationic Lipids for Intracellular Delivery of Biologically Active Molecules filed April 19th, 1990 by Felgner et al., which is hereby incorporated by reference. These cationic

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lipids have the general formula



wherein

$\text{Y}^1$  and  $\text{Y}^2$  are the same or different and are  $-\text{O}-\text{CH}_2-$ ,  $-\text{O}-\text{C}(\text{O})-$ , or  $-\text{O}-$ ;

$\text{R}^1$  and  $\text{R}^2$  are the same or different and are H, or  $\text{C}_1$  to  $\text{C}_{23}$  alkyl or alkenyl;

$\text{R}^3$  and  $\text{R}^4$  are the same or different and are  $\text{C}_1$  to  $\text{C}_{24}$  alkyl, or H;

$\text{R}^5$  is  $\text{C}_1$  to  $\text{C}_{24}$  alkyl straight chain or branched chain;

$\text{R}^6$  is  $-\text{C}(\text{O})-(\text{CH}_2)_m-\text{NH}-$ , a diaminocarboxylic acid which is alkyl, aryl, or aralkyl, or  $-\text{C}(\text{O})-(\text{CH}_2)_m-\text{NH}-$  linked to said diaminocarboxylic acid, or is absent;

$\text{R}^7$  is H, spermine, spermidine, a histone, or a protein with DNA-binding specificity, or wherein the amines of the  $\text{R}_7$  moiety are quaternized with  $\text{R}^3$ ,  $\text{R}^4$ , or  $\text{R}^5$  groups; or

$\text{R}^7$  is an L- or D-alpha amino acids having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine or ornithine or derivatives thereof, or the same amino acids wherein the amine of the  $\text{R}_7$  moiety is quaternized with  $\text{R}^3$ ,  $\text{R}^4$  or  $\text{R}^5$  groups; or

$\text{R}^7$  is a polypeptide selected from the group consisting of L- or D-alpha amino acids, wherein at least one of the amino acids residues comprises arginine, histidine, lysine, ornithine, or derivatives thereof;

$n$  is 1 to 8;

$m$  is 1 to 18; and

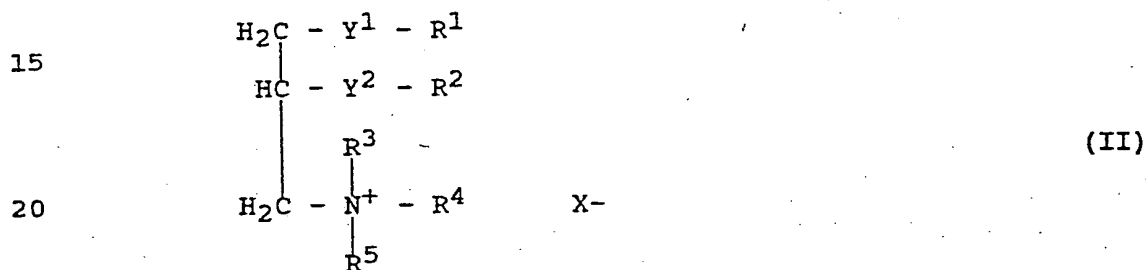
$\text{X}$  is a non-toxic anion.

These compounds have been found to be highly effective for use in lipid formulations for transfection and other

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intracellular delivery procedures. Particularly preferred for *in vivo* transfection or other cell delivery are the diester or ether/ester species of these compounds which are found to be more easily metabolized than previously known cationic lipids while nonetheless retaining a high level of transfective activity. In particularly preferred embodiments, therefore, the positively charged liposomes of the invention comprise the cationic lipids 1,2-dioleoyl-3-dimethylaminopropyl- $\beta$ -hydroxyethylammonium or 1-O-oleyl-, 2-oleoyl-3-dimethylaminopropyl- $\beta$ -hydroxyethylammonium.

For the same reason of metabolizability, ester or ester derivatives of the known cationic lipids having a formula as set forth below are also preferred.



- or an optical isomer thereof, wherein  
 $\text{Y}^1$  and  $\text{Y}^2$  are different and are either  $-\text{O}-\text{CH}_2-$ ,  $-\text{O}-\text{C}(\text{O})-$ , or  $\text{OH}$ ;  
 $\text{R}^1$  and  $\text{R}^2$  are individually absent or are  $\text{C}_1$  to  $\text{C}_{23}$  alkyl or alkenyl;  
 $\text{R}^3$ ,  $\text{R}^4$  and  $\text{R}^5$  are the same or different and are H,  $\text{C}_1$  to  $\text{C}_{14}$  alkyl,  $\text{C}_7$  to  $\text{C}_{11}$  aryl or alkaryl, or at least two of  $\text{R}^3$ ,  $\text{R}^4$ , and  $\text{R}^5$  are taken together to form quinuclidino, piperidino, pyrrolidino, or morpholino;  
 $n$  is 1 to 22; and  
 $\text{X}$  is a non-toxic anion.

Non-toxic anions described herein may be those of pharmaceutically non-toxic acids including inorganic acids and organic acids. Such acids include hydrochloric, hydrobromic, sulfuric, phosphoric, acetic, benzoic, citric, glutamic, lactic acid and the like. For the preparation of



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pharmaceutically acceptable salts, see S. M. Berge et al., Journal of Pharmaceutical Sciences, 66:1-19(1977) which is incorporated herein by reference.

5 In a lipid formulation for preparing positively charged lipid vesicles, the cationic lipid can be present at a concentration of between about 0.1 mole% and 100 mole%, preferably 5 to 95 mole%, and most preferably between 20 and 80 mole%. In a formulation for preparing negatively charged lipid vesicles, the negatively charged  
10 lipid can be present at a concentration between about 0.1 and 100 mole%, preferably 1 to 90 mole%, and most preferably 3 to 50 mole%.

In order to produce lipid vesicles having a net charge, the quantity of the positively or negatively  
15 charged component must exceed that of the alternatively charged component. The alternatively charged lipid can be present at between about 0 to 49 mole% and preferably 0 to 40 mole%.

The neutral lipid can be present in positively or  
20 negatively charged lipid vesicles in a concentration of between about 0 and 99.9 mole %, preferably 5 to 95 mole%, and most preferably 20 to 80 mole%. Cholesterol or a similar sterol can be present at 0 to 80 mole %, and preferably 0 to 50 mole%.

25 The lipid reagents may be prepared and stored as empty liposomes in aqueous solution, or may be stored as dried lipid, for example as a lipid film, after formulation to be later used as encapsulating reagents for selected bioactive substances.

### 30 Liposome Formation

It should be understood that the lipid formulations of the invention, comprising at least one amphipathic lipid, such as a phospholipid, spontaneously assemble to form primary liposomes, heterogeneous in size and  
35 structure, in aqueous solution. Therefore the term lipid reagent, lipid vesicles, and liposomes are used interchangeably in describing formulations.

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The lipid reagents, either negatively or positively charged, are prepared as liposomes or lipid vesicles, according to Example 1. The component lipids are dissolved in a solvent such as chloroform and the mixture evaporated to dryness as a film on the inner surface of a glass vessel. On suspension in an aqueous solvent, the amphipathic lipid molecules assemble themselves into primary liposomes. If other molecules are present in the aqueous solvent, such as, for example, a bioactive substance, these will be captured within the liposomes, as demonstrated in Examples 3 and 5. Alternatively, some lipophilic bioactive substances intended for entrapment can be dissolved or suspended in the organic solvent that is used to dissolve the lipids of the liposome formulation. After evaporation of the lipid film and hydration to form primary liposomes, the substances thus dissolved are typically incorporated into the bilayer of those liposomes, rather than being encapsulated into the aqueous interior. Otherwise, if there is no solute in the hydration buffer, empty liposomes will be formed, as demonstrated in Examples 1 and 2.

To prepare liposomes suitable for physiological in vivo use, having a unilamellar structure and a uniform size of from about 50 to about 200  $\mu\text{m}$  in diameter, the primary liposomes are preferably processed by the freeze-thaw and extrusion processes, as provided by Example 2. These primary liposomes are reduced to a selected mean diameter by means of the freeze-thaw procedure referred to above. The cationic lipids of the invention are formed into vesicles of uniform size prior to transfection procedures, according to methods for vesicle production published in the literature and known to those in the art, for example, the sonication of spontaneously formed liposomes comprised of the lipids in aqueous solution described by Felgner, P.L. et al., Proc. Natl. Acad. Sci., USA 84:7413-7417 (1987) or the reverse-phase evaporation procedure of J. Wilschut et al. Biochemistry 19:6011-6021(1980) or freeze-

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thaw and extrusion (Mayer, L. et al., Biochim. Biophys. Acta 858:161-168 (1986)).

Suitable conventional methods of preparation include, but are not limited to, those disclosed by Bangham, A. et al., J. Mol. Biol. 23: 238-252 (1965); Olson, F. et al., Biochim. Biophys. Acta 557: 9-23 (1979), Szoka F. et al., Proc. Natl. Acad. Sci. USA 75: 4194-4198 (1978), Mayhew, E. et al. Biochim. Biophys. Acta 775: 169-175 (1984), Kim, S. et al. Biochim. Biophys. Acta 728:339-348), and Mayer, L. et al., Biochim. Biophys. Acta 858:161-168 (1986).

According to a preferred method, the negative and positive lipid reagents of the invention, comprising at least one amphipathic lipid species, such as a phospholipid, are prepared using the freeze-thaw-extrusion procedure indicated in Examples 1 and 2. The component lipids are dissolved in a solvent such as chloroform and the mixture evaporated to dryness as a film on the inner surface of a glass vessel. On suspension in an aqueous solvent, the amphipathic lipid molecules assemble themselves into primary liposomes. If other molecules are present in the aqueous solvent, such as, for example, a bioactive substance, these will be captured within the liposomes, as indicated in Examples 3, 4, 5 and 6. Otherwise, empty liposomes will be formed, as in Example 1. These primary liposomes are reduced to a selected mean diameter by means of the freeze-thaw procedure described in Example 2.

The bioactive cargo of the lipid delivery system, according to the primary strategy of the invention, is entrapped into negatively charged liposomes. The lipid derivatives of agents disclosed above, for example, the lipid derivatives of antiviral nucleosides such as phosphatidylazidothymidine or (3'-azido-3'-deoxy)thymidine-5'-diphospho-3-diacylglycerol, can be entrapped by direct incorporation into the wall of the lipid vesicle during the hydration of a lipid film comprising these lipid derivatives in the liposome formulation. Other agents can

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be encapsulated within the aqueous space of the liposome according to conventional liposome forming methodology, as follows.

Encapsulation Procedure

5 Substances intended for intracellular delivery can be encapsulated into negatively or positively charged liposomes by any one of a number of standard procedures. For example, each of the lipid components comprising the liposomal formulation is dissolved together into a co-  
10 miscible organic solvent. If a lipid derivative of an agent to be delivered is to be incorporated into the liposome, a selected quantity of that agent is added to the other lipid components at this time. The solvent is evaporated and the vessel containing the residual lipid  
15 film is evacuated overnight to remove solvent traces.

An hydration solution is next added to the dried lipid film to form primary liposomes. If hydration buffer alone is added to a film of lipid components alone, empty liposomes will form. If hydration buffer alone is added to  
20 a lipid film comprising lipid derivatives of bioactive agent, the primary liposomes will comprise that agent incorporated into the vesicle walls. If hydration buffer having a bioactive dissolved therein is added to a lipid film comprising only lipids, the primary liposomes that  
25 form will comprise that agent trapped in the interior aqueous compartment of the liposomes. Permutations of the processes, resulting in liposomes comprising bioactive agents both incorporated into the walls of the vesicle as well as entrapped therein are possible and are within the  
30 contemplation of the invention.

The hydration solution can be any biologically compatible buffer solution comprising isotonic saline or phosphate buffered saline, or low ionic strength buffers comprising 5% sorbitol or 10% sucrose. Such buffers are  
35 well known to those skilled in the art. The concentration of the bioactive substance in the hydration buffer which is intended for intracellular delivery can vary widely

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depending on the substance or the application; this concentration can be between 1 picogram/ml and 500 mg/ml. Following hydration of the lipid film, the resulting liposome suspension can be further emulsified by any one of  
5 a number of procedures; for example, the sample can be forced through Nuclepore™ membranes to produce vesicles of a size comparable to the pore size of the membranes. Encapsulations are described in Examples 3, 4, and 6 for the encapsulation of FITC dextran, 6-carboxy-fluorescein,  
10 and phalladion in the negatively charged lipid formulation of 4.9/2.1/3 DOPC/DOPG/cholesterol, followed by freeze-thaw and extrusion to achieve a uniform sized liposomal preparation. Any of the substance remaining unencapsulated can be removed, if desired, by a process such as gel  
15 filtration chromatography.

The procedure for delivering materials into cells according to the method of the invention comprises a strategy of presenting the material to the cell in association with positively and negatively charged lipid  
20 vesicles, described as follows, and illustrated by the accompanying Examples.

#### Intracellular Delivery of Liposome Associated Bioactive Substances

In a preferred embodiment of the method of the  
25 invention, following the entrapment of a bioactive substance into negatively charged liposomes, or association of the substance with empty negatively charged liposomes, the resulting liposomes or complexes are added directly to cells, for example in an in vitro application, in a  
30 suitable biologically compatible medium, together with positively charged lipid vesicles. The concentration of lipid with which the cells are contacted varies widely depending on the application, but is between 1  $\mu$ molar and 50 mmolar and preferably from 10  $\mu$ molar to 10 mmolar. The  
35 positively charged lipid vesicles attach spontaneously to the target cell surface, fuse with the cellular membranes and deliver the contents of the liposome into the cell

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cytoplasm.

In an alternate approach, the substance intended for intracellular delivery is first encapsulated into negatively charged liposomes, or complexed with empty negatively charged liposomes, according to the procedures described previously. These primary negative complexes are then exposed to a quantity of positively charged lipid vesicles. Immediately upon mixing the two solutions containing the oppositely charged lipid vesicles, complexes spontaneously form comprising negatively and positively charged vesicles.

The quantity of positively charged vesicles added to the encapsulating negatively charged vesicles should be sufficient to encourage attachment of the complexes to the target cell surface. Theoretically, this quantity should be sufficient to provide the complexes with a net positive charge, with the number of positive charges contributed by the positively charged liposomes in excess over the number of negative charges contributed by the negatively charged liposomes; however, such an excess of positively charged elements is not always required to encourage sufficient attachment to the target cell surface to achieve intracellular delivery. Accordingly, the ratio of positive to negative charges in the final complexes may be from about 100:1 to 0.1:1 and preferably 20:1 to 0.2:1.

#### Intracellular Delivery of Liposome-Associated Polynucleotides

Procedures for the encapsulation and intracellular delivery of polynucleotide using cationic lipid methodology frequently result in a high percentage of the polynucleotide remaining unencapsulated by lipid vesicles. The present invention also contemplates the use of liposome complexes comprising positively charged liposomes but having a net overall negative charge for the intracellular delivery of negatively charged polynucleotides. These complexes are prepared as follows:

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Polynucleotides in solution are mixed with a sufficient quantity of positively charged liposomes so as to form a complex which reduces the anionic characteristics of the polynucleotide by a sufficient amount as described below. Negatively charged elements in a polynucleotide solution can be readily quantified using the extinction coefficient for the nucleotide monomer which bears one negative charge per monomer. The concentration of polynucleotide in solution can be from about 0.01  $\mu\text{g/ml}$  to 50 mg/ml, preferably from 1  $\mu\text{g/ml}$  to 10 mg/ml and most preferably from 10  $\mu\text{g/ml}$  to 1 mg/ml. The concentration of positively charged lipid vesicles can range from between 0.1  $\mu\text{g/ml}$  to 100 mg/ml, preferably from 1  $\mu\text{g/ml}$  to 100 mg/ml, and most preferably from 10  $\mu\text{g/ml}$  to 50 mg/ml. The solutions may be mixed together from those having a low ionic strength buffer, that is having an ionic strength less than that of 25 mM sodium chloride. Sorbitol, sucrose or glucose can be used to render a low ionic strength buffer isotonic. Adsorption of the polyanionic polynucleotides to the cationic vesicles reduces the negative charge character of the polynucleotides. In theory, the quantity of charges contributed by the vesicles should exceed the number of negative charges contributed by the polynucleotide, although this condition may not be an absolute requirement for every application. The ratio of positive to negative charges in the polynucleotide/cationic lipid complexes may be from about 100:1 to 0.1:1 and preferably 20:1 to 0.2:1.

These positively charged complexes can further spontaneously associate with an excess of negatively charged vesicles so as to produce a negatively charged complex containing positively charged lipids and the polynucleotide. By following a protocol of this type, the entire polynucleotide can be incorporated by self-assembly into a lipid complex that can accomplish intracellular delivery without a loss in efficiency caused by incomplete nucleotide encapsulation. In theory, the quantity of

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negatively charged liposomes added should exceed the net positive charge contributed by the complex of positively charged lipid and polynucleotide, although in practice this level of negatively charged elements may not be required for every application. Accordingly, the ratio of positive to negative charges in the polynucleotide/cationic lipid complexes may be 100:1 to 0.1:1 and more preferably 20:1 to 0.2:1.

The negatively charged complexes produced above, containing polynucleotide and positively charged lipids, are analogous to the primary negatively charged lipid complexes disclosed for other bioactive substances, and can be delivered *in vivo* or *in vitro* to tissue culture cells in a manner similar to that described above for those primary complexes.

In a parallel manner, self-assembled systems comprising successive layers of positive and negative liposomes can be manufactured and can be applied to any molecule or composition to be delivered to a cell, regardless of its size or charge. The construction of such structured complexes would be otherwise be extremely difficult.

In the Examples that follow, intracellular delivery of proteins was measured by using signal producing substances that could be visualized in the cells after incorporation. These substances include:

(1) Fluoresceinated dextran which is available in different molecular sizes from 4,000 to 70,000 MW and having a net positive, neutral or negative charge. Delivery of dextran into the cell was quantitated by total cell associated fluorescence as determined by a spectrophotometer. The distribution of fluorescence was determined by fluorescence microscopy. A thin band of fluorescence around the cell was read to indicate cell-associated vesicles; bright spots around the nucleus to indicate lysosomal uptake; and diffuse cytoplasmic fluorescence to indicate cytoplasmic delivery. Cytoplasmic



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delivery is considered the most effective.

(2) Fluoresceinated phalloidin. This 7-amino acid cyclic peptide binds specifically to intracellular actin. Functional delivery of this substance is indicated by the filamentous intracellular staining pattern characteristic of actin filaments seen on examination of the cells by fluorescence microscopy.

(3) Anti-actin antibody. This antibody will bind to actin when delivered intracellularly. Treated cells were fixed, permeabilized and counter-stained with a fluorescent antibody against the anti-actin antibody. As in the case of fluoresceinated phalloidin, effective delivery is indicated by the characteristic actin pattern seen on fluorescence microscopy. Cell viability for all experiments was determined by trypan blue staining.

The results indicate that a variety of biologically significant materials can be delivered by this procedure.

#### Utility

The self-assembling delivery systems of the invention can be advantageously formed using any cationic lipid, whether those previously known, for example, DOTMA or DOTAP, or novel cationic lipids, such as those described above and also disclosed in co-pending U.S. Application of Felgner et al., filed April 19, 1990, to deliver substances intracellularly either in vitro or in vivo. Those cationic lipids described above having metabolizable ester bonds are preferred for in vivo use. In vitro applications include the intracellular delivery to any cell grown in culture, comprising cells of any species, whether plant or animal, vertebrate or invertebrate, and of any tissue or type.

Contemplated uses comprise transfection procedures corresponding to those presently known. Accordingly, the strategies of the invention can be used to facilitate the intracellular delivery of DNA or mRNA sequences coding for therapeutically active polypeptides, as described in detail in U.S. Patent Applications Serial Nos. 326,305 and 467,881 which are hereby incorporated by reference. The self-

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assembling delivery methods described herein however, are particularly preferred for the delivery of the expressed gene product or protein itself. Thus the self-assembling delivery of proteins can provide therapy for genetic disease by supplying deficient or absent gene products to treat any genetic disease in which the defective gene or its product has been identified, such as Duchenne's dystrophy (Kunkel, L. and Hoffman, E. Brit. Med. Bull. 45(3):630-643 (1989) or cystic fibrosis (Goodfellow, P. Nature, 341(6238):102-3 (Sept. 14, 1989).

The self-assembling delivery systems described above can also provide immunizing polypeptides to the cell either by delivering a polynucleotide coding for the immunogen, or the immunogen itself. Other therapeutically important polynucleotides suitable for self-assembling delivery comprise anti-sense polynucleotide sequences, useful in eliminating or reducing the production of a gene product, as described by Ts'o, P. et al. Annals New York Acad. Sci. 570:220-241 (1987).

Also within the scope of the invention is the delivery, by means of the methods disclosed, ribozymes, or catalytic RNA species, either of the "hairpin" type as described by Hampel et al. Nucleic Acids Research 18(2):299-304 (1990); or the "hammerhead" type described by Cech, T. and Bass, B. Annual Rev. Biochem. 55:599-629 (1986).

Particularly preferred within the contemplated uses of the invention are deliveries of either an anti-sense polynucleotide or ribozyme as described above, and having as its target the rev site of the HIV genome (Scientific American, October, 1988, pp. 56-57). Matsukura, M. et al. Proc. Natl. Acad. Sci. 86:4244-4248 (1989) describe a 28-mer phosphorothioate compound anti-HIV (anti-rev transactivator) specific for the site.

The transfection procedures described above may be applied by direct injection of self-assembled lipid complexes, comprising DNA, RNA or proteins into cells of an

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animal *in vivo*. However, these methods are particularly effective at facilitating *in vitro* transfection of cells. Therefore the above therapies can be alternatively carried out by *in vitro* transfection of some of the cells of an animal using self-assembling delivery methods, and re-introduction of the cells into the animal. The ability to transfect cells at high efficiency with self-assembling delivery thus provides an alternate method for immunization. The gene for an antigen is introduced into cells which have been removed from an animal by self-assembling delivery methods. The transfected cells, now expressing the antigen, are reinjected into the animal where the immune system can now respond to the (now) endogenous antigen. The process can possibly be enhanced by co-injection of either an adjuvant or lymphokines to further stimulate the lymphoid cells.

The compositions and methods of the present invention can be most advantageously used in the periodic supplying of endogenous or exogenous macromolecules, particularly proteins, to a cell. They are therefore particularly suitable for use in transient therapies which requires treatment with proteins, particularly in cells unable to carry out translation of messenger RNA.

Examples of such transient therapeutic uses of self-assembling delivery methods herein disclosed include the liposomal delivery of nucleotide analogues having an antiviral effect, such as dideoxynucleosides, didehydronucleosides, nucleoside analogues having halogen substituted and azido-substituted ribose moieties, such as 3'-azido-3'-deoxythymidine (AZT), or nucleoside analogues such as acyclovir or gancyclovir (DHPG). Lipid derivatives of antiviral nucleosides are presented in U.S. Patent Applications Serial Nos. 216,412; 319,485; and 373,088 which are hereby incorporated by reference. These lipid derivatives are conveniently incorporated into the vesicle walls of the primary negatively charged liposomes according to the primary strategy of the invention.

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Among other therapeutically important agents that can be thus delivered are peptides comprising physiologic species such as interleukin-2, tumor necrosis factor, tissue plasminogen activator, factor VIII:C, erythropoietin, growth factors such as epidermal growth factor, neural growth factor, and hormones such as tissue insulin, calcitonin, and human growth hormone as well as toxic peptides such as ricin, diphtheria toxin, or cobra venom factor, capable of eliminating diseased or malignant cells.

Also within the scope of the invention is the intracellular delivery of antiviral formulation through topical application of the lipid complexes disclosed comprising acyclovir or gancyclovir for the treatment of Herpes simplex. These formulations preferably comprise lipid derivatives of the antiviral agents, particularly the phosphatidylglycerol derivatives as disclosed in U.S. Applications Serial Nos. 216,412, 319,485 and 373,088. The effect of the self-assembled lipid delivery system is to facilitate the penetration of the active antiviral agent through the stratum corneum of the dermis.

Self-assembling systems comprising lipid-encapsulated bioactive agents may accordingly be used for intracellular delivery either *in vivo* or *in vitro*. For *in vitro* applications, neutral or positively charged bioactive molecules are encapsulated in a negatively charged lipid reagent and added to a washed cell culture together with a volume of a suspension of liposomes comprising the positively charged lipid reagent, as indicated in Example 8. For *in vivo* applications, corresponding molecules are encapsulated with negatively charged lipid reagent and then coated with the liposomes of the positively charged lipid reagent before administration.

#### EXAMPLE 1: Positively Charged Liposome Preparation

Lipids used: 1,2-Bis(oleoyloxy)-3-(trimethylammonio) propane, DOTAP; dioleoyl phosphatidylethanolamine, DOPE.

A quantity of 5 mg of DOTAP was combined with 5 mg

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DOPE in 1 ml chloroform, dried in a Rotovap and the flask evacuated overnight on a vacuum pump. The lipids were then suspended in 0.5 ml double deionized water, and sonicated in a bath sonicator for 30 minutes at 15° or until the suspension was clear. A clear solution is indicative of liposome formation. (When the lipid concentration is greater than 80 mg/ml the solution does not clear.)

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#### EXAMPLE 2: Empty Negatively Charged Liposomes

Lipids used: dioleoylphosphatidylcholine, DOPC; dioleoylphosphatidylglycerol, DOPG; cholesterol

Quantities of 4.67 mg DOPC, 4.06 mg DOPG and 2.9 mg cholesterol comprising 25 uMoles of total lipid, in a molar ratio of 4.9/2.1/3, DOPC/DOPG/cholesterol, were combined in 1 ml of chloroform. The mixture was dried on a Rotavap apparatus and the flask evacuated overnight on a vacuum pump.

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The lipid film was suspended at 20°C for 1 hour using a rotary shaker with 1 ml tris buffer (20 mM pH 7.5) and the suspension adjusted to 290 mOsm by adding crystalline NaCl. The sample was frozen in a dry ice/isopropanol bath and thawed in a 30°C bath. The sample was then extruded under 200-750 psi using two stacked 0.2 micron polycarbonate filter membranes (Nuclepore™) in a 10 ml volume "Extruder" (Lipex Biomembranes Inc., Vancouver, Canada). The freeze/thaw and extrusion was repeated two more times. The final product was extruded two more times.

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#### EXAMPLE 3: Encapsulation of FITC Dextran in Negatively Charged Liposomes

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A quantity of 200 mg FITC Dextran (Sigma Chemical Co.) was dissolved in 1.5 ml 20 mM Tris buffer (pH 7.5) and the solution adjusted to 290 mOsm with NaCl. A lipid film, comprising 25 uMoles of total lipid, in a molar ratio of 4.9/2.1/3, DOPC/DOPG/Cholesterol, was prepared as in Example 2. The lipid film at 20°C was suspended in 1.5 ml

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of the FITC Dextran solution for 1 hour using a rotary shaker. The sample was frozen in a dry ice/isopropanol bath and thawed in a 30°C bath, then extruded under 200-750 PSI through stacked 0.2 micron polycarbonate filter membranes (Nuclepore™) in a 10 ml volume "Extruder" (Lipex Biomembranes Inc., Vancouver, Canada). The freeze/thaw and extrusion was repeated two more times. The final product was extruded two more times. The sample was applied to a Sephadex G-75 column equilibrated with the 20 mM tris buffer (adjusted to 290 mOsm with NaCl) and the void volume, which contains the liposome encapsulated FITC dextran, collected.

EXAMPLE 4: Rhodamine-Phosphatidylethanolamine Labeled Negatively Charged Liposomes

A quantity of 22 mg egg phosphatidylcholine, 9.3 mg egg phosphatidylglycerol, 7.7 mg cholesterol and 1 mg of rhodamine-phosphatidylethanolamine were dissolved into 1 ml of chloroform. The lipid film was suspended at 20°C for 1 hour using a rotary shaker with Dulbecco's phosphate buffered saline (PBS) pH 7.4. The sample was frozen in a dry ice/isopropanol bath and thawed in a 30°C bath. The sample was extruded under 200-750 psi using two stacked 0.1 micron polycarbonate filter membranes (Nuclepore™) in a 10 ml volume "Extruder" (Lipex Biomembranes Inc., Vancouver, Canada). The freeze/thaw and extrusion was repeated two more times. The final product was extruded two more times.

EXAMPLE 5: Encapsulation of 6 carboxy-fluorescein in Negatively Charged Liposomes

A quantity of 23.5 mg 6 carboxy-fluorescein (6CF) was placed into 2.5 ml of 20 mM phosphate buffer (pH 7.4); and approximately 160 ul of 1N NaOH added to dissolve 6CF. The solution was readjusted to pH 7.4; and adjusted to 290 mOsm with NaCl. This solution contained 8.8 mg/ml 6CF (23.4 mM).

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A quantity of 9.34 mg DOPC, 8.12 mg DOPG and 5.8 mg cholesterol was placed into 2 ml of chloroform, comprising 50  $\mu$ M of total lipid in a ratio of 4.9/2.1/3 DOPC/DOPG/Cholesterol. The lipid film was suspended at 20°C for 1 hour using a rotary shaker with 0.75 ml of the 6CF solution. The sample was frozen in a dry ice/isopropanol bath and thawed in a 30°C bath. The sample was then extruded under 200-750 psi using two stacked 0.2 micron polycarbonate filter membranes (Nuclepore) in a 10 ml volume "Extruder" (Lipex Biomembranes Inc., Vancouver, Canada). The freeze/thaw and extrusion was repeated two more times. The final product was extruded two more times. The sample was frozen in a dry ice isopropanol bath and thawed at room temperature. It was then extruded through 0.2 micron nucleopore filter membranes. The freeze/thaw and extrusion was repeated two more times. The final product was extruded two more times. The sample was applied to a Sephadex G-75 column equilibrated with the 20 mM tris buffer (adjusted to 290 mOsm with NaCl) and the void volume, containing the liposome encapsulated 6CF collected.

EXAMPLE 6: Encapsulation of Phalladion in Negatively Charged Liposomes

A volume of 3 ml of methanolic phalloidin solution (3.3  $\mu$ M) was dried on the Rotovap. The phalloidin peptide was dissolved into 0.5 ml of Tris buffer (20 mM pH 7.5) and adjusted to 290 mOsm).

A quantity of 4.67 mg DOPC, 4.06 mg DOPG and 2.9 mg cholesterol was taken up into 1 ml of chloroform. This results in 25 uMoles of total lipid, in a molar ratio of 4.9/2.1/1/3, DOPC/DOPG/Cholesterol. The lipid film was suspended at 20° C for 1 hour using a rotary shaker with 1.5 ml of the phalladion solution. The sample was frozen in a dry ice/isopropanol bath and thawed in a 30°C water bath. The sample was then extruded under 200-750 psi using two stacked 0.2 micron polycarbonate filter membranes (Nuclepore™) in a 10 ml volume "Extruder" (Lipex

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Biomembranes Inc., Vancouver, Canada). The freeze/thaw and extrusion was repeated two more times. The dried lipid film was suspended in 3 ml of the phalloidin solution. The sample was frozen in a dry ice/isopropanol bath and thawed at room temperature. It was then extruded through 0.2 micron nucleopore filter membranes. The freeze/thaw and extrusion was repeated two more times. The final product was extruded two more times. The sample was then applied to a Sephadex G-75 column equilibrated with the 20 mM tris buffer (adjusted to 290 mOsm with NaCl) and the void volume, containing the liposome encapsulated phalloidin collected.

#### 15 EXAMPLE 7: Cells

PBS (Dulbecco's phosphate buffered saline without calcium or magnesium), DMEM (Dulbecco's minimum essential medium) were obtained from Irvine Scientific (Irvine, CA.). Opti-MEM was obtained from Gibco Laboratories (Life Technologies, Inc., Grand Island, New York). Bovine calf serum was obtained from Hyclone. Trypsin, EDTA (0.5 gm trypsin, 0.2 gm EDTA/liter) in Hank's salts was from Irvine Scientific.

25 NIH 3T3 cells, a contact inhibited mouse fibroblast line, are from Dr. Marguerite Vogt, Salk Institute. The cells are grown in monolayer in DMEM + 10% calf sera, 1% fungibact (Irvine Scientific). Cells are removed by trypsinization with trypsin-EDTA solution. Cells are seeded onto 4 cm<sup>2</sup> Lab-Tek tissue culture cells per well. NIH 3T3 cells are maintained in subconfluent stock cultures to retain their contact inhibition.

HELA cells are a human carcinoma epithelial cell line. The cells are maintained in DMEM + 10% fetal calf sera + 1% fungibact. The cells grow by attachment to a surface and trypsinized for transfer. Cells are seeded at low density for microscope slides at approximately 1000 cells per 4 cm<sup>2</sup>.



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EXAMPLE 8: Delivery of Detectable Substances into Cells  
Using Self-assembling Charged Lipid Complexes

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Positively charged liposomes, prepared as described in Example 1 were diluted with water to give a concentration of 1.15 ng/ul. Negatively charged liposomes, comprising FITC dextran, 6-carboxy fluorescein, or phalladion, 10 encapsulated in a negatively charged lipid vesicle and prepared according to Examples 4, 5 and 6 respectively, were diluted to give a concentration of approximately 0.05 ng/ul with Tris buffer (20 mM, pH 7.5; 290 mOsm).

15 The substances were then delivered into either HeLa or 3T3 cells as follows:

Growing media was removed from the cells which were then washed with phosphate buffered saline (PBS) 2 times. Opti-Mem non-serum media (2 ml) was then to each 4 cm<sup>2</sup> well. Quantities of 35 ul of empty positively charged lipid 20 vesicles, prepared according to Example 1 and 80 ul of negatively charged lipid vesicles having the detectable substances indicated above encapsulated within were added to each of the wells containing cells. Plates were incubated for 2 hours at 37°C, then washed with PBS 2 times 25 and viewed with the epi-fluorescent microscope using the appropriate filters.

RESULTS:

30 Comparing phase contrast photomicrograph (a) with the corresponding fluorescence photomicrograph (b) in each case:

Figure 1 shows intracytoplasmic delivery of fluoresceinated dextran (MW 4000) encapsulated into 35 negatively charged vesicles, into NIH-3T3 cells according to the methods previously described, using DOTMA/DOPE vesicles. This dye is considered to be a mimic for a water soluble peptide, and its behavior would be expected to be indicative of peptide delivery. Figure 2 gives similar 40 results with HELA cells.

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A low molecular weight fluorescent dye, 6-carboxy fluorescein, encapsulated into negatively charged vesicles can also be delivered into 3T3 cells using DOTMA/DOPE by this method as indicated on Figure 4. Again the comparison with the light micrograph illustrated co-localization with the cytoplasm of the cells.

Figure 5 shows the results of delivery of negatively charged vesicle encapsulated Rhodamine-phalladion, into NIH-3T3 cells using DOTAP/DOPE. Fluorescence is confined to the cytoplasm in these micrographs and the pattern of fluorescence is typical of phalladion binding to intracellular actin filaments.

Similarly the lipophilic dye, Rhodamine-PE, prepared as indicated in Example 6, can also be delivered intracellularly by this method, into NIH-3T3 cells using DOTMA/PE vesicles (Figure 3). Comparison with the light photomicrograph indicates a concentration of fluorescence intracellularly.

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#### EXAMPLE 9: A Kit for Intracellular Delivery Procedures

A kit was constructed to provide materials to accomplish the delivery and uptake of macromolecules into tissue culture cells. The materials in the kit consisted of a vial containing dried lipid, a buffer solution, a solution containing cationic lipid vesicles, a syringe provided with a 0.4 micron Nuclepore™ membrane in a filter housing, and an instruction leaflet. The lipid vial contained 35 mg of 1-palmitoyl-2-oleoyl-phosphatidylcholine and 15 mg of dioleoylphosphatidylglycerol in a dried lipid film. The buffer solution consisted of 130 mM NaCl and 10 mM sodium phosphate buffer at a pH of 7.4.

The kit was used to deliver monoclonal antibodies to intercellular actin for transfection into mammalian cells according to the instructions provided. A quantity of the macromolecule to be delivered, in this case, 1 mg of the actin antibody, was delivered into the buffer solution and mixed well. The solution was then placed in the lipid-

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containing vial to hydrate the lipid film and form a liposome suspension. The lipid suspension was then loaded into the barrel of the syringe provided, and the liposome suspension was forced out through the filter membrane to  
5 form smaller lipid vesicles of a more uniform size. Under these conditions approximately 10% of the initial quantity of macromolecule is encapsulated into the lipid vesicles.

The cationic lipid vesicle solution provided in the kit consisted of 0.5 mg/ml of a DORI diether and 0.5 mg/ml  
10 dioleoylphosphatidylethanolamine. The user is instructed to combine 5 $\mu$ l of the negatively charged liposomes with 100  $\mu$ l of the DORI/DOPE solution and to add the mixture onto tissue culture cells (approximately 10<sup>6</sup> cells) growing on OptiMem™ media without serum. The incubation of the cells  
15 then continues for 2 hours. The cells are washed, and then assayed for the biological endpoint, as in Example 8.

There will be various modifications, improvements, and applications of the disclosed invention that will be apparent to those of skill in the art, and the present  
20 application is intended to cover such embodiments. Although the present invention has been described in the context of certain preferred embodiments, it is intended that the full scope of the disclosure be measured by reference to the scope of the following claims.

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WHAT IS CLAIMED IS:

1. A method for making a vehicle for administering a bioactive substance to a cell, comprising the steps of:

5       providing said substance in a first lipid vesicle, said vesicle comprising at least one negatively charged lipid and having a net negative charge; and

10       combining said first lipid vesicle with second lipid vesicles, said vesicles comprising at least one cationic lipid and having a net positive charge; whereby said first lipid vesicles are coated with at least one positively charged lipid vesicle.

15       2. The method of Claim 1, wherein said bioactive substance is lipophilic or comprises lipophilic regions, and is thereby incorporated into the lipid structure of said first lipid vesicle.

3. The method of Claim 1, wherein said bioactive substance is encapsulated within said first lipid vesicle.

20       4. A method for making a vehicle for administering a bioactive substance to a cell, comprising the steps of:

25       contacting a bioactive substance with at least one lipid vesicle, said lipid vesicle comprising a negatively charged lipid species and having a net negative charge, whereby said bioactive substance and said lipid vesicle form a first lipid complex; and

30       contacting said first lipid complex with lipid vesicles comprising at least one cationic lipid and having a net positive charge, whereby said first lipid complex and said positively charged lipid vesicles form a second lipid complex.

5. The method of Claim 4 wherein said bioactive substance is lipophilic or has lipophilic regions.

35       6. The method of Claim 4, wherein said bioactive substance has positively charged regions or has a net positive charge.

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7. The method of any one of Claims 1 through 6, further comprising the steps of:

- 5 (a) contacting said vehicle with negatively charged lipid vesicles to form a lipid complex having negatively charged regions on its outer surface; and  
(b) contacting said lipid complex with positively charged lipid vesicles to form a vehicle having positively charged regions on its outer surface.

8. The method of Claim 7, further comprising  
10 repeating steps (a) and (b) at least once.

9. A method for administering a bioactive substance to a cell, comprising the steps of:

- providing said substance in a negatively charged lipid vesicle;  
15 administering said negatively charged complex to a cell together with lipid vesicles comprising at least one cationic lipid, said vesicles having a net positive charge.

10. A method for administering a bioactive substance  
20 to a cell, comprising the steps of:

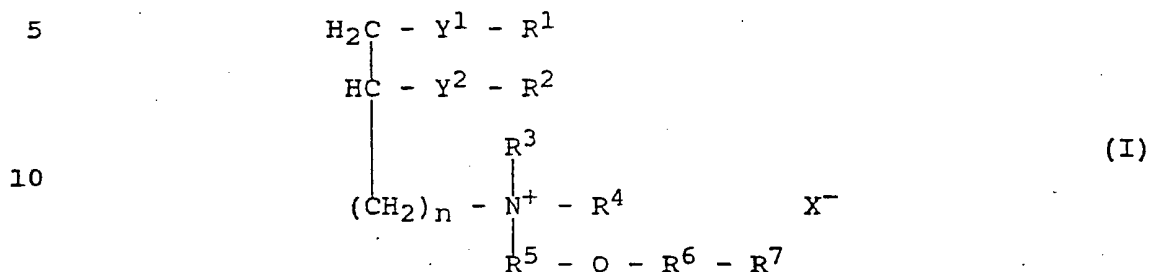
- preparing a complex comprising said bioactive substance and a negatively charged lipid, said complex having a net negative charge;  
administering said negatively charged complex to  
25 a cell together with lipid vesicles comprising at least one cationic lipid, said vesicles having a net positive charge.

11. The method of Claim 9 or 10 wherein said bioactive substance is administered in vivo.

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12. The method of any one of Claims 1 through 6 wherein said cationic lipid has the structure



15 wherein

$\text{Y}^1$  and  $\text{Y}^2$  are the same or different and are  $-\text{O}-\text{CH}_2-$ ,  $-\text{O}-\text{C}(\text{O})-$ , or  $-\text{O}-$ ;

20  $\text{R}^1$  and  $\text{R}^2$  are the same or different and are H,  $\text{C}_1$  to  $\text{C}_{23}$  alkyl or alkenyl;

$\text{R}^3$  and  $\text{R}^4$  are the same or different and are  $\text{C}_1$  to  $\text{C}_{24}$  alkyl, or H;

$\text{R}^5$  is  $\text{C}_1$  to  $\text{C}_{24}$  alkyl straight chain or branched chain;

25  $\text{R}^6$  is  $-\text{C}(\text{O})-(\text{CH}_2)_m-\text{NH}-$ , a diaminocarboxylic acid which is alkyl, aryl, or aralkyl, or  $-\text{C}(\text{O})-(\text{CH}_2)_m-\text{NH}-$  linked to said diaminocarboxylic acid, or is absent;

30  $\text{R}^7$  is H, spermine, spermidine, a histone, or a protein with DNA-binding specificity, or the same groups wherein the amines of the  $\text{R}^7$  moiety are quaternized with  $\text{R}^3$ ,  $\text{R}^4$ , or  $\text{R}^5$  groups; or

$\text{R}^7$  is a L- or D-alpha amino acid having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine or ornithine or derivatives thereof, or the same amino acids wherein the amine of the  $\text{R}^7$  moiety is quaternized with  $\text{R}^3$ ,  $\text{R}^4$  or  $\text{R}^5$  groups; or

40  $\text{R}^7$  is a polypeptide selected from the group consisting of L- or D-alpha amino acids, wherein at least one of the amino acids residues comprises arginine, histidine, lysine, ornithine, or derivatives thereof;

$n$  is 1 to 8;

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m is 1 to 18; and  
 X is a non-toxic anion,  
 providing that when n is 1,  
 R<sup>5</sup> is -CH<sub>2</sub>-CH<sub>2</sub>-, R<sup>6</sup> is absent, and R<sup>7</sup> is H, then R<sup>1</sup> and R<sup>2</sup>  
 5 are not both stearyl.

13. A method according to Claim 12 wherein said cationic lipid is DL-1,2-dioleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium and its salts.

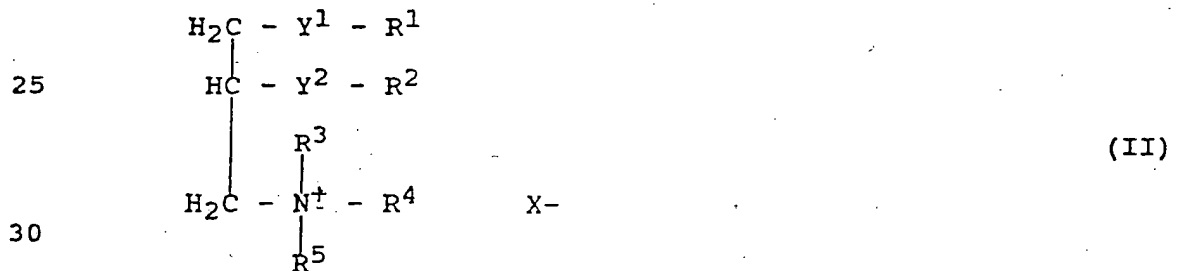
14. A method according to Claim 12 wherein y<sup>1</sup> and y<sup>2</sup>  
 10 are different and are either -O-CH<sub>2</sub>- or -O-C(O)-.

15. A method according to Claim 14 wherein at least one cationic lipid is 1-O-oleyl-, 2-oleoyl-3-dimethylaminopropyl-β-hydroxyethylammonium.

16. A method according to any one of Claims 1 through  
 15 6, wherein said cationic lipid is 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP).

17. A method according to any one of Claims 1 through  
 6 wherein said cationic lipid is an  
 N-(ω, ω-1-dialkoxy)-alk-1-yl-N, N, N-trisubstituted  
 20 ammonium surfactant.

18. A method according to Claims 1 through 6 wherein at least one cationic lipid has the structure



or an optical isomer thereof, wherein  
 R<sup>1</sup> and R<sup>2</sup> are individually absent, C<sub>1</sub> to C<sub>23</sub> alkyl or  
 35 alkenyl, or H;  
 y<sup>1</sup> and y<sup>2</sup> are different and are either -O-CH<sub>2</sub>-, -O-C(O)- or  
 OH;  
 R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are the same or different and are H, C<sub>1</sub> to  
 C<sub>14</sub> alkyl, C<sub>7</sub> to C<sub>11</sub> aryl or alkaryl, or at least two of  
 40 R<sup>3</sup>, R<sup>4</sup> and R<sup>5</sup> are taken together to form quinuclidino,

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piperidino, pyrrolidino, or morpholino;

n is 1 to 22; and

X is a non-toxic anion.

19. A composition of matter, comprising:

5 a bioactive substance contained in at least one negatively charged lipid vesicle; and

lipid vesicles comprising at least one cationic lipid species and having a net positive charge at least partially covering said negatively charged lipid vesicle.

10 20. The composition of Claim 19 wherein said bioactive substance is encapsulated within said negatively charged lipid vesicle.

21. The composition of Claim 19 comprising a bioactive substance which is lipophilic or has a lipophilic region whereby said bioactive substance is incorporated among the lipids of said negatively charged lipid vesicle.

22. A composition of matter comprising a primary complex of a bioactive substance and at least one negatively charged lipid vesicle; and

lipid vesicles comprising at least one cationic lipid species and having a net positive charge at least partially covering said negatively charged lipid vesicle to form a secondary complex.

25 23. The composition of Claim 22 wherein said bioactive substance comprises positively charged regions or has a net positive charge.

24. The composition of Claim 19 or 22, wherein said composition has a net positive charge.

30 25. The composition of Claim 19 or 22 wherein said bioactive substance is a protein or polypeptide.

26. The composition of Claim 19 or 22 wherein said bioactive substance is a polynucleotide.

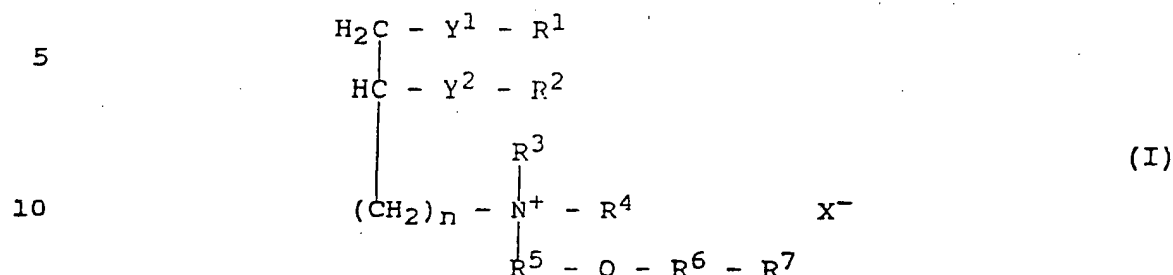
35 27. The composition of Claim 26 wherein said bioactive substance is an antisense polynucleotide.

28. The composition of Claim 19 or 22 wherein said bioactive substance is a drug.



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29. The composition of any one of Claims 19 through 27 wherein at least one cationic lipid has the structure



wherein

Y<sup>1</sup> and Y<sup>2</sup> are the same or different and are -O-CH<sub>2</sub>-, -O-C(O)-, or OH;

R<sup>1</sup> and R<sup>2</sup> are the same or different and are H, C<sub>1</sub> to C<sub>23</sub> alkyl or alkenyl, or are absent;

R<sup>3</sup> and R<sup>4</sup> are the same or different and are C<sub>1</sub> to C<sub>24</sub> alkyl, or H;

R<sup>5</sup> is C<sub>1</sub> to C<sub>24</sub> alkyl straight chain or branched chain;

R<sup>6</sup> is -C(O)-(CH<sub>2</sub>)<sub>m</sub>-NH-, a diaminocarboxylic acid which is alkyl, aryl, or aralkyl, or -C(O)-(CH<sub>2</sub>)<sub>m</sub>-NH- linked to said diaminocarboxylic acid, or is absent;

R<sup>7</sup> is H, spermine, spermidine or a histone or the same groups wherein the amine is quaternized with R<sup>3</sup>, R<sup>4</sup>, or R<sup>5</sup> groups; or

R<sup>7</sup> is an L- or D-alpha amino acid having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine or ornithine or the same amino acids wherein the amine is quaternized with R<sup>3</sup>, R<sup>4</sup> or R<sup>5</sup> groups, or polypeptides comprising said L- or D-alpha amino acids;

n is 1 to 8;

m is 1 to 18; and

X is a non-toxic anion,

providing that when n is 1,

R<sup>5</sup> is -CH<sub>2</sub>-CH<sub>2</sub>-, R<sup>6</sup> is absent, and R<sup>7</sup> is H, then R<sup>1</sup> and R<sup>2</sup> are not both stearoyl.

30. The composition of Claim 29, wherein at least one cationic lipid is

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1,2-dioleoyl-3-dimethylaminopropyl- $\beta$ -hydroxyethylammonium.

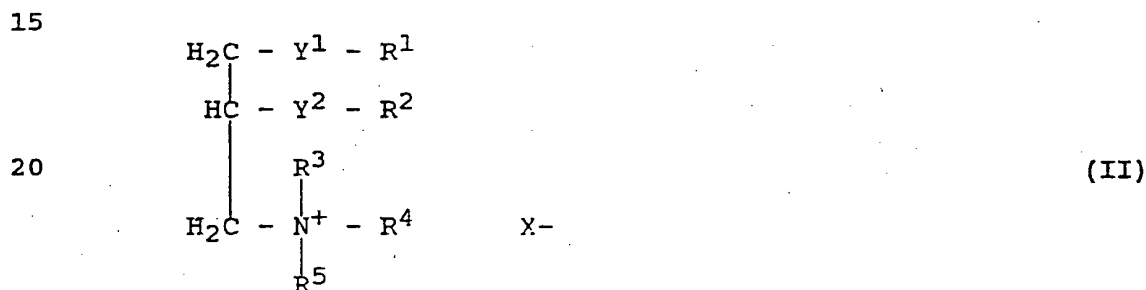
31. The composition of Claim 29, wherein  $Y^1$  and  $Y^2$  are different and are either  $-O-CH_2-$  or  $-O-C(O)-$ .

32. The composition of Claim 31, wherein at least one  
5 cationic lipid is 1-O-oleyl-, 2-oleoyl-3-dimethylaminopropyl- $\beta$ -hydroxyethylammonium.

33. The composition of Claim 29, wherein said cationic lipid is 1,2-bis(oleoyloxy) 3-(trimethylammonio)-propane (DOTAP).

10 34. The composition of Claim 29, wherein said cationic lipid is an N-( $\omega$ ,  $\omega$ -1-dialkoxy)-alk-1-yl-N, N, N-trisubstituted ammonium surfactant.

35. The composition of Claim 29, wherein at least one cationic lipid has the structure



25

or an optical isomer thereof, wherein

$R^1$  and  $R^2$  are individually absent,  $C_1$  to  $C_{23}$  alkyl or alkenyl;

30  $Y^1$  and  $Y^2$  are different and are either  $-O-CH_2-$ ,  $-O-C(O)-$  or OH;

$R^3$ ,  $R^4$  and  $R^5$  are the same or different and are H,  $C_1$  to  $C_{14}$  alkyl,  $C_7$  to  $C_{11}$  aryl or alkaryl, or at least two of  $R^3$ ,  $R^4$  and  $R^5$  are taken together to form quinuclidino, piperidino, pyrrolidino, or morpholino;

35 n is 1 to 22; and

X is a non-toxic anion.

36. A kit for preparing self-assembling lipid complexes for the delivery of a bioactive substance to a cell, either *in vitro* or *in vivo*, comprising:

40 at least one vessel containing at least one

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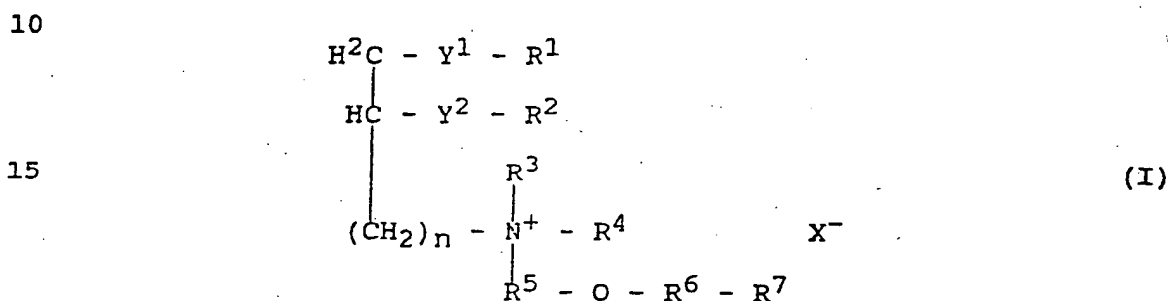
negatively charged lipid species;

a quantity of a lipid substance, said substance comprising at least one cationic lipid species.

37. The kit of Claim 36, further comprising at least  
5 one hydrating buffer solution.

38. The kit of Claim 36, further comprising a membrane filter device.

39. The kit of Claim 36, wherein said cationic lipid species has the structure



20 wherein

$\text{Y}^1$  and  $\text{Y}^2$  are the same or different and are  $-\text{O}-\text{CH}_2-$ ,  $-\text{O}-\text{C}(\text{O})-$ , or  $\text{OH}$ ;

$\text{R}^1$  and  $\text{R}^2$  are the same or different and are H,  $\text{C}_1$  to  
25  $\text{C}_{23}$  alkyl or alkenyl, or are absent;

$\text{R}^3$  and  $\text{R}^4$  are the same or different and are  $\text{C}_1$  to  $\text{C}_{24}$  alkyl, or H;

$\text{R}^5$  is  $\text{C}_1$  to  $\text{C}_{24}$  alkyl straight chain or branched chain;

$\text{R}^6$  is  $-\text{C}(\text{O})-(\text{CH}_2)_m-\text{NH}-$ , a diaminocarboxylic acid which is  
30 alkyl, aryl, or aralkyl, or  $-\text{C}(\text{O})-(\text{CH}_2)_m-\text{NH}-$  linked to said diaminocarboxylic acid, or is absent;

$\text{R}^7$  is H, spermine, spermidine or a histone or the same groups wherein the amine is quaternized with  $\text{R}^3$ ,  $\text{R}^4$ , or  $\text{R}^5$  groups; or

35  $\text{R}^7$  is an L- or D-alpha amino acids having a positively charged group on the side chain, said amino acids comprising arginine, histidine, lysine or ornithine or the same amino acids wherein the amine is quaternized with  $\text{R}^3$ ,  $\text{R}^4$  or  $\text{R}^5$  groups, or polypeptides comprising said L- or D-  
40 alpha amino acids;

$n$  is 1 to 8;

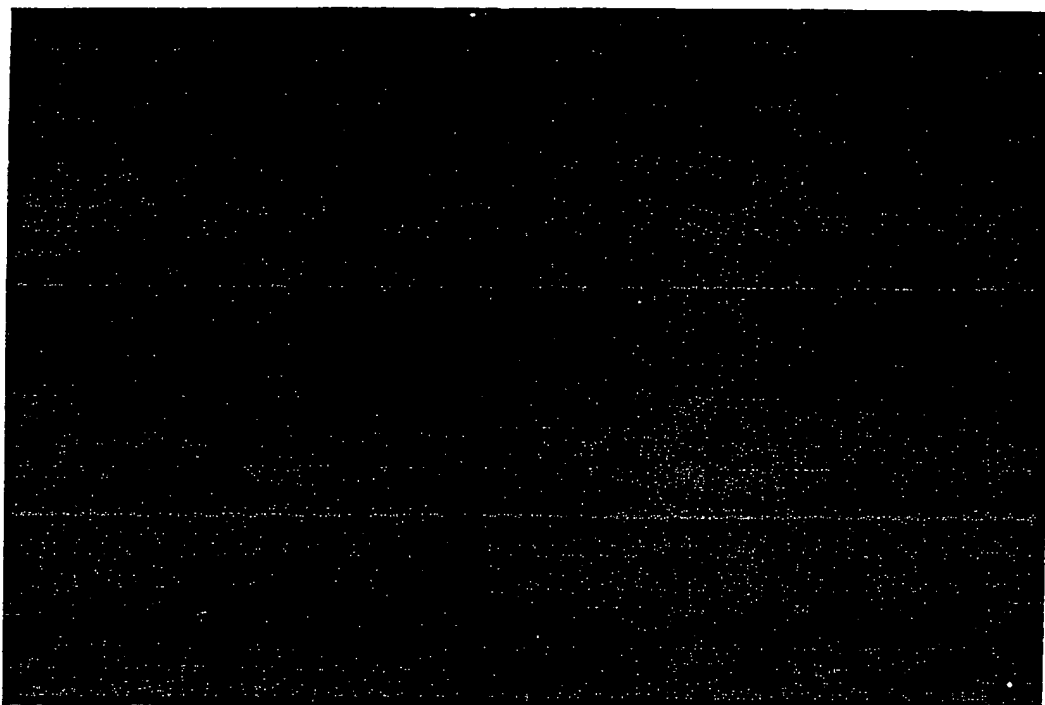
-42-

m is 1 to 18; and  
X is a non-toxic anion,  
providing that when n is 1,  
R<sup>5</sup> is -CH<sub>2</sub>-CH<sub>2</sub>-, R<sup>6</sup> is absent, and R<sup>7</sup> is H, then R<sup>1</sup> and R<sup>2</sup>  
5 are not both stearyl.

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**FIG. 1(a)**



**FIG. 1(b)**

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FIG.2(a)

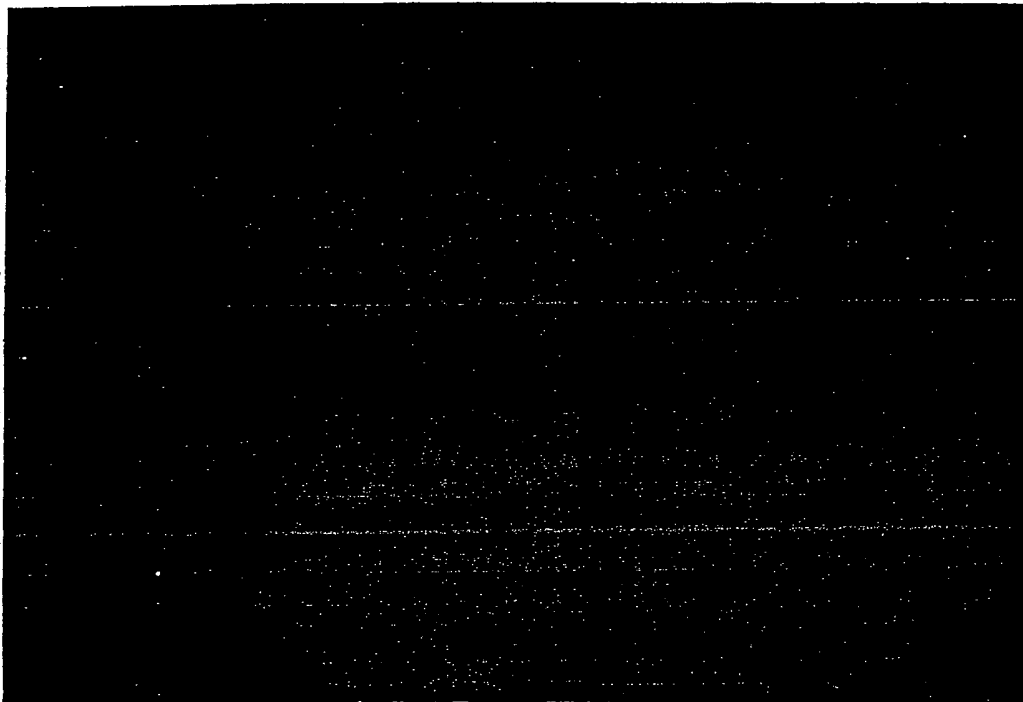


FIG.2(b)

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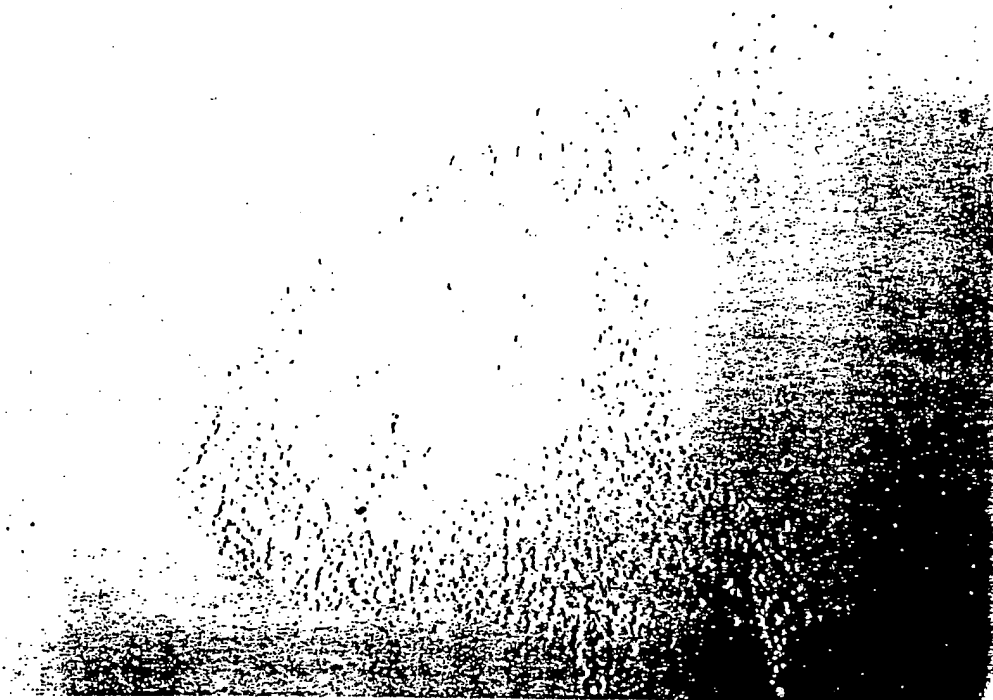


FIG.3(a)

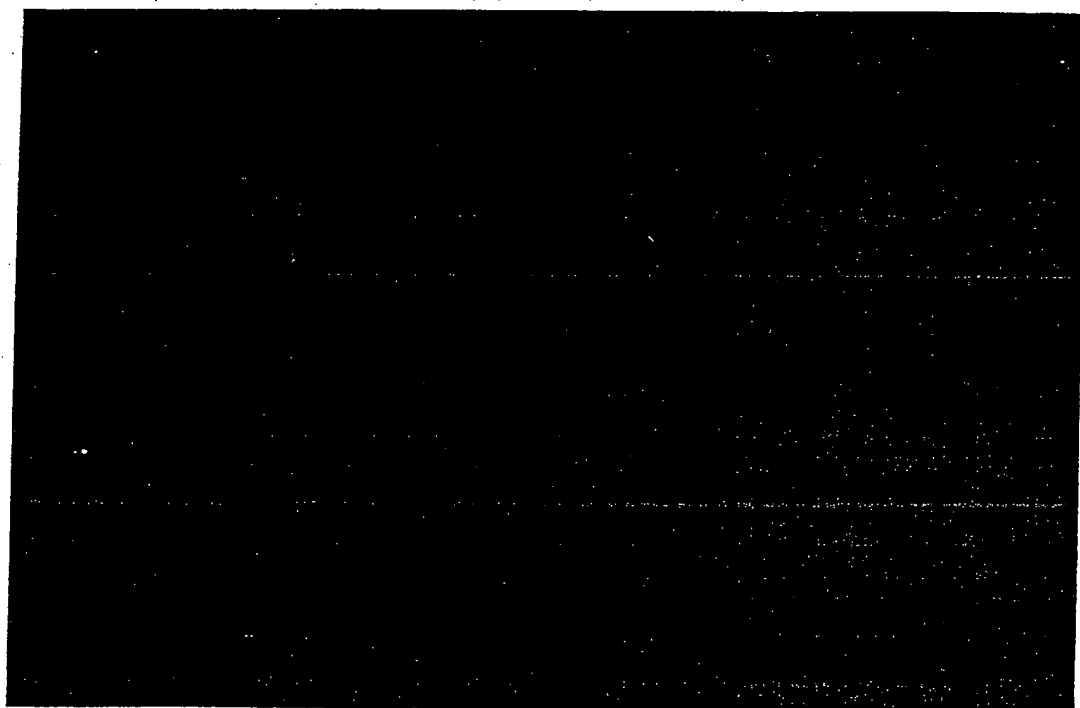


FIG.3(b)

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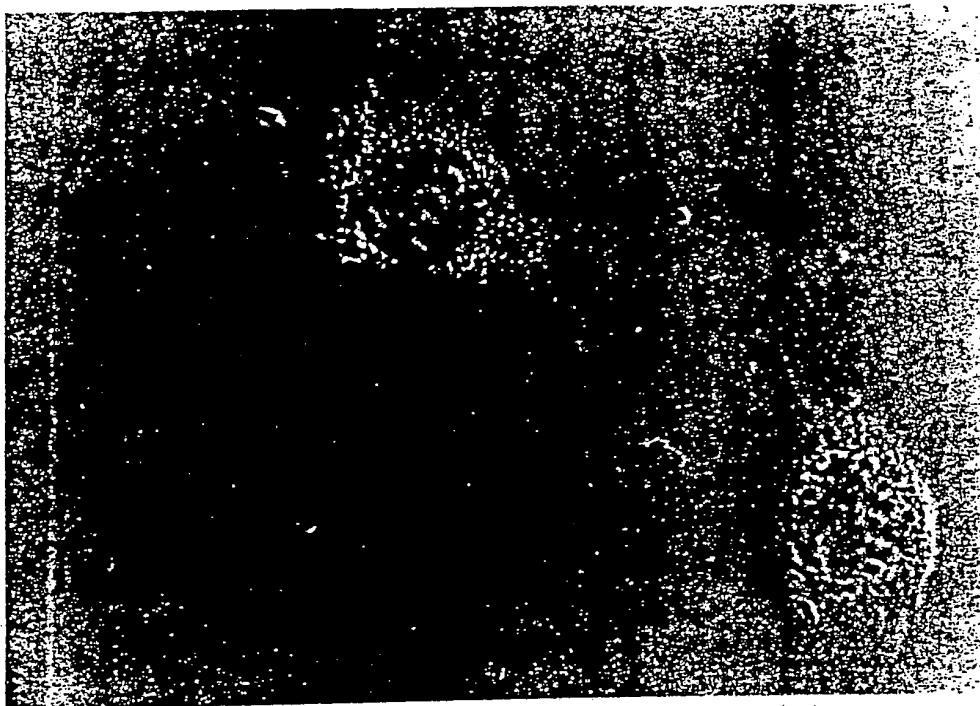


FIG.4(a)

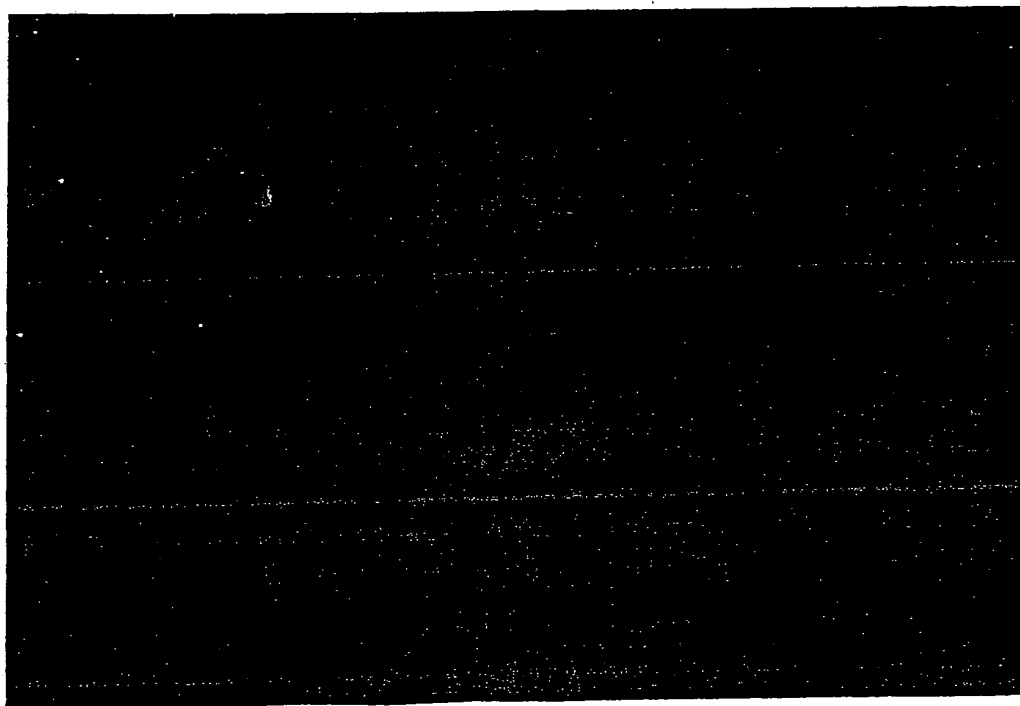


FIG.4(b)



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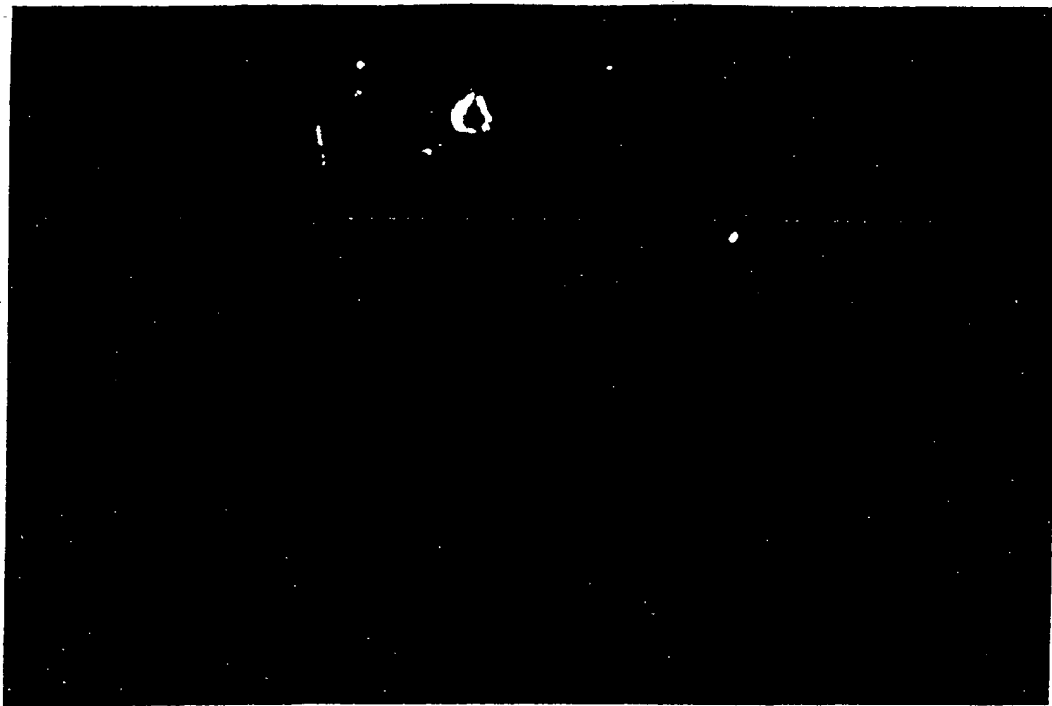


FIG. 5(a)

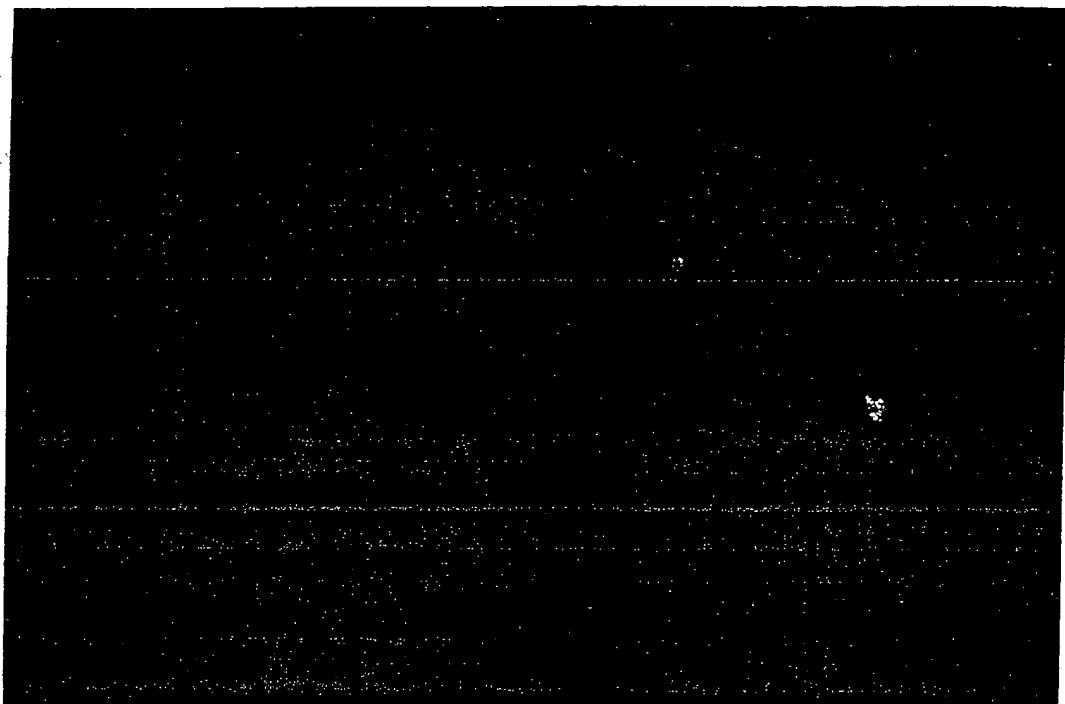


FIG. 5(b)

# INTERNATIONAL SEARCH REPORT

International Application

PCT/US91/02962

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classifications apply, indicate all)		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): G01N 21/00,33/92; C12N 5/00; A01N 37/18		
US.CL.: 422/61; 435/240.2; 436/71; 514/2		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>1</sup>		
Classification System	Classification Symbols	
U.S. CL.	422/61; 435/240.2; 436/71; 514/2	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>2</sup>		
APS, CAS		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>3</sup></b>		
Category <sup>4</sup>	Citation of Document, <sup>5</sup> with indication, where appropriate, of the relevant passages <sup>6</sup>	Relevant to Claim No. <sup>3</sup>
X	US, A, 4,897,355 (EPPSTEIN et al.)	9-11
Y	30 January 1990, see entire document.	19-35
Y	US, A, 4,485,054 (MEZFI et al.)	1-35
	27 November 1981, see entire document.	
Y	Biochemistry, volume 27, issued 1988, I. Stamatatos et al., "Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes", pages 3917-3925, see entire document.	1-35
Y	Biochimica et Biophysica acta, volume 858, issued 1986, L.D. Mayer et al., "Vesicles of variable sizes produced by a rapid extrusion procedure", pages 161-168, see entire document.	1-35
<p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
26 June 1991		03 SEP 1991
International Searching Authority		Signature of Authorized Officer
ISA/US		Bennett Celsa
		BENNETT CELSA
		BG-8-16-91

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A US, A, 4,774,085 (EIDIFR) 27 September 1988. 26-10  
see entire document.

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>12</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

(See Attachment)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims: \_\_\_\_\_
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: \_\_\_\_\_
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Attachment  
PCT/US91/02962  
PCT/ISA/210, Section VI:

I. Claims 1-8 are drawn to a 1st method of making a vehicle for administering a bioactive substance to a cell: Class 436, subclass 71 and Class 514, subclass 2.

II. Claims 9-11 are drawn to a 2nd method of administering a bioactive substance to a cell: Class 424, subclass 450 and Class 514, subclass 2.

III. Claims 12-18 are drawn to a 3rd method of making a vehicle for administering a bioactive substance using a cationic lipid: Class 435, subclass 240.2.

IV. Claims 19-35 are drawn to a composition containing a bioactive substance and a cationic lipid vesicle: Class 435, subclass 240.2.

V. Claims 36-39 are drawn to a kit for self-assembling liquid complexes: Class 422, subclass 61.

The claims of these five groups are drawn to distinct inventions as shown by their different classifications and which are not linked so as to form a single general inventive concept. PCT Rule 13.1 and 13.2 do not provide for multiple products and

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